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<120> Thermostable kanamycin nucleotidyltransferase and screening method of thermophiles using thereof

<130> RJH11-072T

<160> 11

<170> PatentIn Ver. 2.0

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50 55 60

Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val Asn Phe Tyr
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Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp
85 90 95

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Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
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Gln Lys Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
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Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
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Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
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Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr Asp His Leu
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Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
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Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Arg Val Glu Ser Asp Trp
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Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
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Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
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Thr Glu Ala Val Lys Gln Pro Asp Leu Pro Ser Gly Tyr Asp His Leu
195 200 205

Cys Gln Leu Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
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His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe
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Gln Lys Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
165 170 175

Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
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Thr Glu Ala Val Lys Gln Pro Asp Leu Pro Pro Gly Tyr Val Gln Leu
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Cys Gln Leu Val Met Ser Gly Gln Leu Ser Asp Pro Glu Lys Leu Leu
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Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp
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ccg ctt aca cat ggt caa ttt ttc tct att ttg ccg att tat gat tca 336
Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile Tyr Asp Ser
100 105 110

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Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
115 120 125

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Gln Thr Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
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Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu

180 185 190

act gaa gca gtt aag caa tca gat ctt cct tca ggt tat gac cat ctg 624
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Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
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20 25 30

Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr

35 40 45

Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Glu Ala Glu Phe
50 55 60

Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val Asn Phe Asp
65 70 75 80

Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp
85 90 95

Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile Tyr Asp Ser
100 105 110

Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
115 120 125

Gln Thr Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
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Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
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Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
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Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
180 185 190

Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr Asp His Leu
195 200 205

Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
210 215 220

Glu Ser Leu Glu Asn Phe Trp Asn Gly Ile Gln Glu Trp Thr Glu Arg
225 230 235 240

His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe
245 250

[Sequence Listing Free Text]

SE ID NO:1: mutant enzyme obtained by introduction of point mutation into wild type KNT gene of Staphylococcus aureus and its expression

SE ID NO:2: mutant enzyme obtained by introduction of point mutation into wild type KNT gene of Staphylococcus aureus and its expression

SE ID NO:3: mutant enzyme obtained by introduction of point mutation into wild type KNT gene of Staphylococcus aureus and its expression

SE ID NO:4: 5'-Primer for PCR amplification

SE ID NO:5: 3'-Primer for PCR amplification

SE ID NO:6: 5'-Primer for PCR amplification

SE ID NO:7: 5'-Primer for subcloning of WT*

SE ID NO:8: 5'-Primer for subcloning of KT3-11 and HTK

SE ID NO:9: 3'-Primer for subcloning

[Brief Description of the Drawings]

[Figure 1]

Figure 1 indicates the restriction maps for each of plasmids (a)pYK134, (b)pTT8, and (c)pJHK1.

[Figure 2]

Figure 2 indicates the thermostability of KNT.

(A) The heat denaturation of WT* (□), KT3-11 (△) and HTK (○) was monitored on a CD at 222nm and recorded. Measurement conditions were: protein concentration 0.8 μ M; 50mM potassium phosphate buffer containing 0.1M KCl; and pH of 7.0.

Degree of Denaturation (%) = $(\theta^{T}_{222} - \theta^{N}_{222})/(\theta^{D}_{222} - \theta^{N}_{222})$

(θ^{T}_{222} is the average residue molecular ellipticity at T℃, at 222nm and θ^{N}_{222} and θ^{D}_{222} are the average residue molecular ellipticities at 222nm for the non-denatured and denatured enzyme, respectively.)

(B) Heat Inactivation: Enzyme solution is heated for ten minutes at the designated temperature. After cooling, activity is measured at 25℃. Heat treatment was conducted with a protein concentration of 1.2 μ M and using the same buffer as in the CD measurement. The values for each enzyme are expressed as a ratio in comparison to the respective non-heat-treated enzyme. Each numerical value has a standard deviation of ±10%

[Figure 3]

Figure 3 indicates a 3-dimensional representation of the structure of KNT having the Asp80Tyr mutation (KT3-11 and HTK). KNT is a homodimer, and the positions and the residue numbers of modified residues are indicated for only one of the subunits. The mutated residues of KT3-11, the additional 9 mutated residues of HTK, kanamycin, and adenosine 5'- α, β -methylene triphosphate which is an analog of ATP, are indicated. This figure was prepared using MOLSCRIPT (Per Kraulis, Department of Molecular Biology, Uppsala University, Sweden.)

[Figure 4]

Figure 4 indicates a restriction map for plasmid pJHK3.

[Designation of Document] Abstract

[Problem to be solved]

To obtain a selective marker suitable for screening of thermophilic bacteria such as *Thermus thermophilus*. *T. thermophilus* are good research materials for investigating the interrelation between enzyme structures and functions since they are stable at extreme pH, crystallize easily and are easy-to-handle.

[Means for Solving the Problem]

A novel kanamycin nucleotidyltransferase with markedly improved thermostability, a selective marker using the same, and a screening method for thermophilic bacteria such as *Thermus thermophilus* using said selective marker, are provided.

[Representative Drawing] Figure 2.